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Aristolochic acid impedes endocytosis and induces DNA adducts in proximal tubule cells

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Background. Aristolochic acid (AA), present in *Aristolochia* plants, appears to be the toxin responsible for Chinese herbs nephropathy (CHN), a rapidly progressive tubulointerstitial nephritis. One of the earliest sign of CHN is the urinary excretion of low-molecular-weight proteins (LMWP), suggesting that AA is toxic to proximal tubules (PT).

Methods. The effects of AA on PT functions including reabsorption of LMWP were investigated on the well-established opossum kidney (OK) cell line, a model for PT, and compared with those of the classical PT toxin cadmium chloride ($CdCl_2$).

Results. OK cell monolayers internalized albumin and β_2-microglobulin by receptor-mediated endocytosis, both proteins apparently competing for the same receptor, a complex of megalin and cubulin. The process was significantly impaired by 24-hour preincubation with AA (10 or 20 μmol/L) or $CdCl_2$ (15 μmol/L). Furthermore, 24-hour exposure to AA followed by its removal during one to six days led to a persistent inhibition of the uptake of albumin, in contrast to the substantial recovery observed after $CdCl_2$ removal. Neither AA nor $CdCl_2$ affected cell viability, Na^+-glucose cotransport or total rate of protein synthesis. AA significantly decreased megalin expression and formed specific DNA adducts in OK cells, similar to those found in kidneys from CHN patients.

Conclusions. The present data support the involvement of AA in the early PT dysfunction found in CHN; furthermore, they suggest a causal relationship between DNA adduct formation, decreased megalin expression, and inhibition of receptor-mediated endocytosis of LMWP.

Aristolochic acid (AA) is the active principle extracted from *Aristolochia* plants, consisting essentially of a mixture of structurally related nitrophenanthrene derivatives AAI and AAII [1]. Acute AA intoxication in rodents leads to acute renal failure, whereas chronic administration induces multisystemic tumors [2, 3]. Recently, ingestion of AA has been incriminated in the outbreak of the so-called "Chinese herbs nephropathy" (CHN), a severe tubulointerstitial nephritis initially described in Belgium in the early 1990s [4], but now also reported in other European [5–7] and Asian countries [8, 9]. CHN was initially encountered in young women attending a single obesity clinic where they followed a slimming diet containing Chinese herbs. Because of a manufacturing error, one of the herbs was inadvertently replaced by *Aristolochia fangchi*, which contains nephrotoxic AA [10]. In addition to a rapidly progressive interstitial renal failure due to a particularly severe fibrosis [11, 12], the clinical course of CHN is complicated by tumoral transformations in the urothelium [13, 14].

One of the most consistent features of CHN is the early occurrence of tubular proteinuria consisting chiefly of β_2-microglobulin and albumin [15], occasionally associated with normoglycemic glycosuria [16] and neutral endopeptidase enzymuria [17]. This tubular proteinuria, detected in asymptomatic patients well before any change in serum creatinine levels [15], suggested that impairment of proximal tubule (PT) function might be an early manifestation of AA toxicity in the kidney. That hypothesis is supported by previous toxicological studies in rodents, which showed that administration of massive amounts of AA induces acute and selective PT lesions [2] and also by renal biopsies from patients with CHN, which showed regenerative tubular epithelia mainly along PT profiles [11, 12].

A key function of the PT is to reabsorb plasma proteins that escape into the glomerular filtrate as a function of their size and charge [reviewed in 18]. These low-molecular-weight proteins (LMWP) below 42 Å (or 70 kD) include β_2-microglobulin, α_1-microglobulin, vitamin D-bind-

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ing protein, retinol-binding protein or Clara cell protein, with albumin lying at the extreme upper limit. Filtered albumin and LMWP are almost totally reabsorbed along the PT, which explains why urinary protein level is below 300 mg/day [19]. When PT function is compromised, a "tubular proteinuria" appears, consisting of LMWP with some albumin [19, 20]. Recent studies have shown that protein reabsorption by the PT essentially occurs via receptor-mediated endocytosis, a complex process that involves binding to a receptor located within the luminal membrane of epithelial cells followed by endocytosis of the protein-receptor complex, catabolism of the protein within lysosomes, and recycling of the receptor to the luminal membrane [21–23]. Pathological damage to the PT, with ensuing tubular proteinuria, thus might be the first noticeable manifestation of AA toxicity.

This hypothesis was tested in the opossum kidney (OK) cell line, a classic model for the study of various transport processes occurring in the PT [24]. OK cells share numerous morphological and functional properties with PT epithelial cells, including Na^+-glucose cotransport [25], Na^+-H^+ exchange mediated by the NHE type III isoform [26], specific transport of organic anions and cations [27, 28], and Na^+-phosphate cotransport regulated by parathyroid hormone [29]. In particular, OK cells display a remarkable ability to reabsorb proteins by receptor-mediated endocytosis, a process that involves the megalin and cubilin receptors previously identified in PT cells [30, 31]. In the present study, we used OK cells as an in vitro model of the PT to investigate the potential nephrotoxicity of AA in comparison with that of cadmium chloride ($CdCl_2$), a potent nephrotoxin known to impair PT functions [32, 33].

METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM)-F12, DMEM without L-methionine and L-glutamine, and other reagents for cell culture were obtained from Life Technologies (Merelbeke, Belgium). $CdCl_2$, AA (a mixture of AAI and AAII, predominantly AAI), ethylenediaminetetraacetic acid (EDTA), 3-(4,5-dimethyl-thiazoyl-2-yl)-2,5diphenyltetrazolium bromide (MTT), cycloheximide, dodecyl sulfate sodium salt (SDS), tetramethylrhodamine isothiocyanate (TRIC)-labeled anti-sheep, anti-β-actin were from Sigma (Bornem, Belgium). The lactate dehydrogenase (LDH) Cytotoxicity Detection kit was from Roche Diagnostics (Brussels, Belgium). Paraformaldehyde and Triton X-100 were from Boehringer Ingelheim (Verviers, Belgium). Fluorescein-isothiocyanate (FITC)-labeled bovine albumin was purchased from Molecular Probes (Leiden, The Netherlands); β_2-microglobulin from ICN Biomedicals (Asse-Relegem, Belgium); Cy™5 labeling and enhanced chemiluminescence (ECL) kits were from Amersham Pharmacia Biotech (Roosendaal, The Netherlands); Bicinchoninic acid (BCA) Protein Assay Reagent kit from Pierce (Polylab, Antwerpen, Belgium); [^{35}S]L-methionine (>800 Ci/mmol), [^{14}C]methyl-α-D-glucopyranoside ([^{14}C]AMG) from NEN Life Science Products (Zaventem, Belgium); peroxidase-labeled anti-sheep and anti-mouse antibodies from Dako (Prosan, Merelbeke, Belgium); all other chemicals were from Merck-Belgolabo (Overijse, Belgium).

Cell culture and treatment

Opossum kidney cells (Dr. G. Friedländer, Université Xavier-Bichat, Paris, France) of passage number 46 to 108 were grown in DMEM-F12 medium containing 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were incubated at 37°C in air containing 5% CO_2. Confluent OK cells were treated 24 hours with 0.1% dimethyl sulfoxide (DMSO), 10 or 20 μmol/L AA in 0.1% DMSO, and 15 μmol/L $CdCl_2$ in water or left untreated. Alternatively, OK monolayers were incubated for 24 hours with 20 μmol/L AA or 15 μmol/L $CdCl_2$, followed by a recovery period (1 to 6 days) in culture medium alone. In another set of experiments, exposure to treatment was shortened to 15 or 30 minutes or to 2, 6, 14, 18 hours.

Assessment of cytotoxicity and cell viability in OK cells treated with AA or $CdCl_2$ for 24 hours was performed with the LDH Cytotoxicity Detection kit and MTT assay [34], following the recommendations of the manufacturers.

Uptake of albumin and β_2-microglobulin

The uptake experiments were performed as described previously [30]. After washing, the OK cells were incubated with either FITC-albumin or Cy5-β_2-microglobulin in Ringer's solution at 37°C for 15 minutes. The intracellular fluorescence was measured at the excitation and emission wavelength at 492 and 520 nm, respectively, for FITC-albumin or 649 and 670 nm for Cy5-β_2-microglobulin. Protein content was measured by the BCA protein assay.

OK cell morphology and confocal microscopy examination

For morphological examination, OK cells on glass coverslips were fixed for ten minutes with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, at room temperature, counterstained with hematoxylin-eosin, and observed under a DMR microscope coupled to a MPS60 photomicrographic system (Leica, Heerbrugg, Switzerland). For functional studies of endocytosis, OK cell monolayers were incubated at 37°C for 15 minutes with Ringer's solution containing FITC-albumin (150 μmol/L) and then placed on ice, rinsed, and fixed as described previously in this article prior to observation under a

Zeiss Axiovert fluorescence microscope coupled to a Laser-Scanning Confocal Microscope (MRC 1000; Bio-Rad, Hercules, CA, USA) equipped with an argon-krypton laser and with Laser-Sharp software (Bio-Rad). Images were further analyzed using the NIH-image 1.62 software (Bethesda, MD, USA).

For colocalization studies of megalin and endocytosis, OK cells were incubated at 37°C for 2, 5, and 15 minutes with FITC-albumin (150 μmol/L) before fixation and immunostaining with a sheep anti-megalin antibody (1:200 dilution) [35, 36]. After washing, the cells were incubated with a TRITC-labeled anti-sheep antibody (diluted 1:200), washed and viewed under confocal imaging system as described previously in this article. The confocal settings were defined to avoid signal interferences between the two fluorescent channels. All experiments were performed in duplicate and compared with those performed in absence of primary antibody as controls.

Radiolabeling of proteins in OK cells with [^{35}S]L-methionine

Opossum kidney cell monolayers, treated for 24 hours with AA or $CdCl_2$, or for one hour with cycloheximide (100 μmol/L), were exposed to [^{35}S]L-methionine ~20 μCi/mL in methionine-free DMEM for two hours. After washing, monolayers were incubated for 1 or 20 hours at 37°C, 5% CO_2 in DMEM supplemented with 10 mmol/L methionine. After washing, the cells were lysed (20 min on ice in Tris 10 mmol/L, EDTA 1 mmol/L, pH 8). Lysates proteins, precipitated by trichloroacetic acid (10% for 30 minutes on ice), were collected by centrifugation (5000 rpm at 4°C for 20 minutes). The pellets were resuspended in 1% SDS and counted for β-emission (1214 Rackbeta liquid scintillation counter, LKB Wallac, Finland).

Uptake of methyl-α-D-glucopyranoside (AMG)

After washing, OK cells were incubated with [^{14}C]-AMG in Ringer's solution at 37°C for ten minutes (that is, during the linear phase of the uptake). After rinsing with ice-cold Ringer's solution, the cells were lysed by 0.1 mol/L HNO_3 and counted for β-emission.

^{32}P-postlabeling analysis of DNA from OK cells

Aristolochic acid-DNA adducts were determined by the nuclease P1-enrichment method of the ^{32}P-postlabeling assay [37]. Briefly, DNA was isolated from OK cells by the phenol extraction method after trypsinization of two culture flasks in each condition studied. DNA samples (12.5 μg) were digested, enriched, and ^{32}P postlabeled as described [37]. Subsequently samples were chromatographed on PEI-cellulose thin layer sheets (Macherey and Nagel, Düren, Germany). Chromatographic conditions used are as follows: D1, 1 mol/L sodium phosphate, pH 6.8; D3, 3.5 mol/L lithium formate, 8.5 mol/L urea, pH 4.0; D4, 0.8 mol/L LiCl, 0.5 mol/L Tris-HCl, 8.5 mol/L urea, pH 9.0; D5, 1.7 mol/L NaH_2PO_4, pH 6.0. Aristolochic acid-DNA adducts were identified as described previously [38] and quantitated using an Instant Imager (Canberra Co., Dowers Grove, IL, USA).

Immunoblot analyses

Cell lysates were prepared from confluent OK cells [35]. After washing in PBS and gentle scraping, cells were centrifuged at 8000 × *g* for 90 seconds, and the pellet was frozen in liquid nitrogen. Frozen pellets were solubilized in ice-cold lysis buffer containing protease inhibitors (Complete Mini®; Roche Diagnostics, Brussels, Belgium), briefly sonicated (Branson Sonifier 250, 2 pulses at 40% intensity), and then centrifuged at 16,000 × *g* for one minute at 4°C. The supernatant was transferred into tubes containing 10% SDS and heated at 95°C for 90 seconds.

Protein extracts were separated by SDS-polyacrylamide gels and transferred to nitrocellulose. After blocking, membranes were incubated overnight at 4°C with sheep anti-megalin antibodies (1:2500 dilution) [36], washed, incubated with peroxidase-labeled anti-sheep antibody, and visualized with ECL. For reprobing, the membranes were rinsed, incubated for 30 minutes at 55°C in a stripping buffer (62.5 mmol/L Tris-HCl, 2% SDS, 100 mmol/L β-mercaptoethanol, pH 7.4), washed again, and incubated with ECL to verify the absence of signal (1-hour exposure). The membranes were then incubated with a monoclonal antibody against β-actin (1:10,000 dilution) and thereafter with peroxidase-labeled anti-mouse antibody, before exposure to ECL. Three independent immunoblotting experiments for megalin were performed in OK cells.

Densitometry analysis was performed on a representative blot with a Hewlett Packard Scanjet model IVC using the NIH Image V1.60 software (Bethesda, MD, USA). The optical densities for megalin (given as arbitrary pixel units) were normalized against β-actin and expressed in the percentage of the signal obtained in control OK cells. Each determination was obtained in duplicate.

Statistical analysis

All data are expressed as mean ± SEM. Comparisons were performed by paired Student *t* test analysis.

RESULTS

Receptor-mediated endocytosis of albumin and β_2-microglobulin in OK cells

The uptake of FITC-albumin by OK cells followed Michaelis-Menten kinetics at 37°C characteristic of receptor-mediated endocytosis (Fig. 1A) [30]. The low rate of uptake observed at 4°C most probably reflected the albumin remaining bound to the cells despite thorough washing. The uptake of β_2-microglobulin followed similar

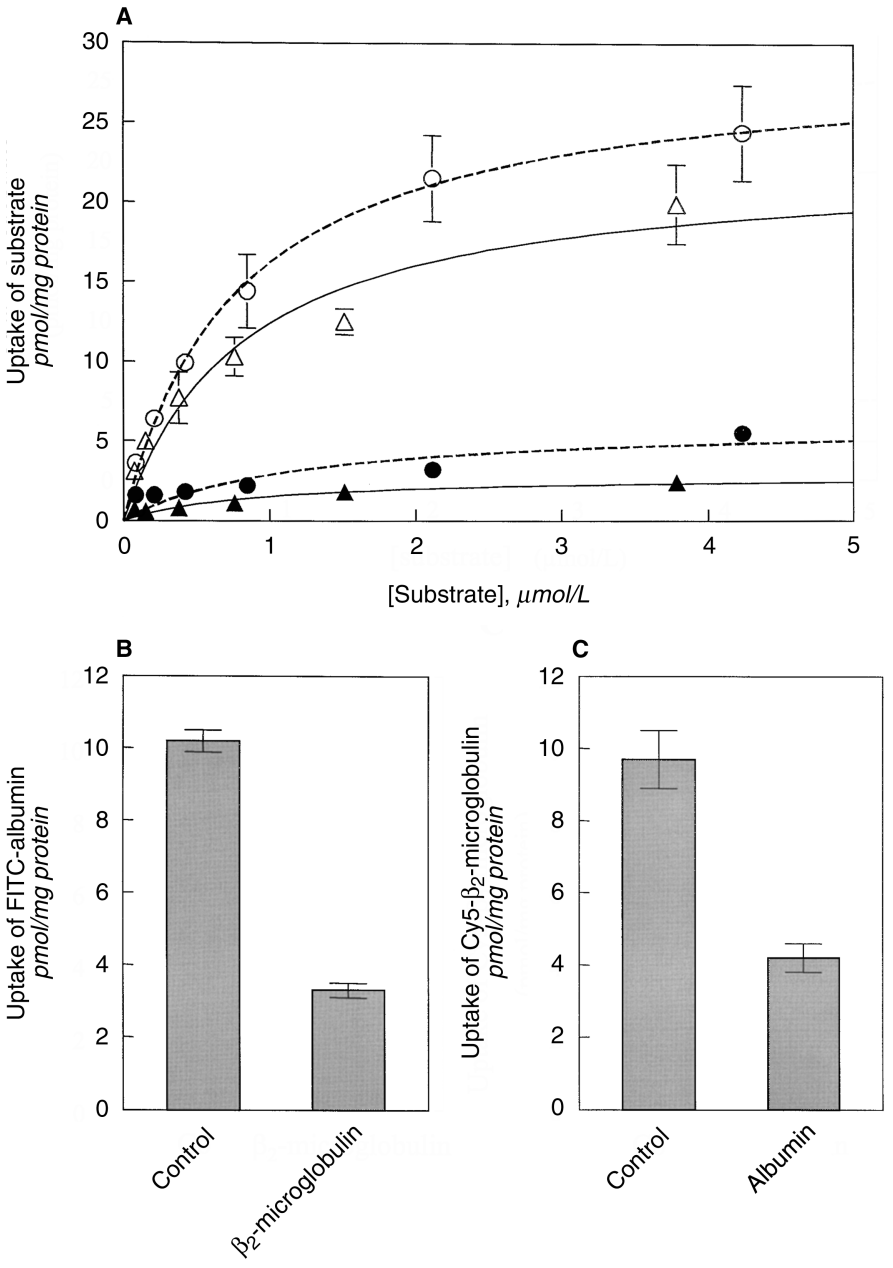


Fig. 1. (*A*) Initial rate (15 minutes) of uptake of FITC-albumin (triangles) and Cy5-β_2-microglobulin (circles) as a function of substrate concentrations in Ringer's solution either at 37°C (open symbols) or on ice (filled symbols). (*B* and *C*) Competition between β_2-microglobulin and albumin for receptor-mediated endocytosis. (B) Inhibition of the uptake of FITC-albumin (0.8 μmol/L) by a 100-fold excess of β_2-microglobulin; (C) inhibition of the uptake of Cy5-β_2-microglobulin (0.8 μmol/L) by a 100-fold excess of albumin. Each column represents the difference between the values observed at 37°C and at 4°C ($N = 3$).

Michaelis-Menten kinetics (Fig. 1A). As shown, β_2-microglobulin significantly interferes with albumin uptake (Fig. 1B) and vice versa (Fig. 1C). This finding suggests that both proteins compete for the same membrane receptor in OK cells.

Both AA and $CdCl_2$ inhibit protein reabsorption in OK cells

As shown in Figure 2A and 2B, the uptake of albumin and β_2-microglobulin was noticeably reduced by a 24-hour preincubation of the OK cell monolayers with 10 or 20 μmol/L AA. Furthermore, after a 24-hour exposure of the monolayers to 20 μmol/L AA followed by a 24-hour recovery period, the uptake of both albumin and β_2-microglobulin remained inhibited (Fig. 2). Pretreatment of OK monolayers with 15 μmol/L $CdCl_2$ for 24 hours also markedly reduced the uptake of both albumin and β_2-microglobulin (Fig. 3). The magnitude of that inhibitory effect was similar to that observed with AA. When the uptake curves were analyzed in terms of Michaelis-Menten kinetics (Table 1), both $CdCl_2$ and AA caused a significant decrease in V_{max} (the decrease being dose-dependent for AA exposure) but left K_m unchanged except in one case.

To determine the time course of inhibition precisely, short exposures to AA and $CdCl_2$ were performed. For

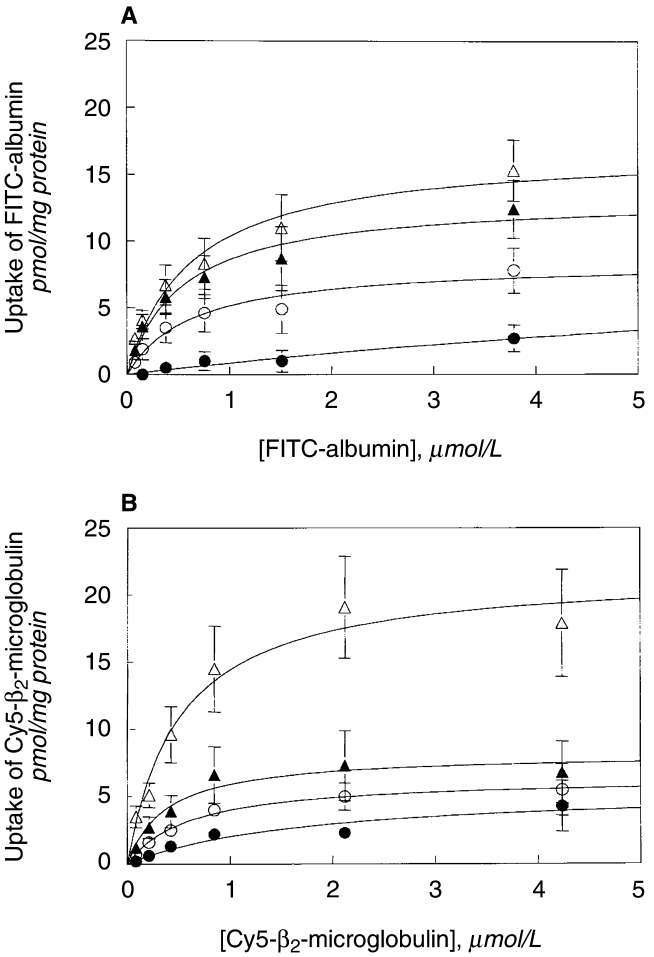


Fig. 2. Effect of aristolochic acid (AA) on the initial uptake of FITC-albumin (*A*) and Cy5-β_2-microglobulin (*B*). OK cells were preincubated with 0.1% DMSO as control (△), 10 μmol/L (▲), and 20 μmol/L (○) AA for 24 hours, and with 20 μmol/L AA for 24 hours followed by a 24-hour recovery period in the culture medium (●). Each point represents the difference between the values observed at 37°C and at 4°C ($N = 3$). The statistics are given in Table 1.

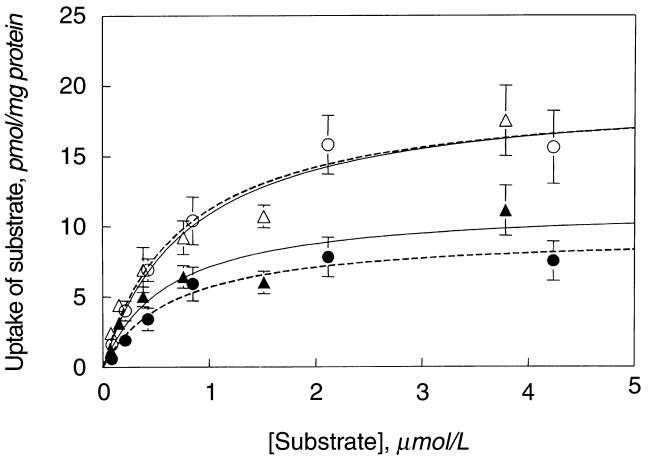


Fig. 3. Effect of cadmium chloride ($CdCl_2$) on the initial uptake of FITC-albumin (triangles) and Cy5-β_2-microglobulin (circles). The cells were preincubated with 15 μmol/L $CdCl_2$ for 24 hours (filled symbols) or left untreated (open symbols). Each point represents the difference between the values observed at 37°C and at 4°C ($N = 3$). The statistics are given in Table 1.

AA, a progressive inhibition was observed, reaching significance after 14 hours. In contrast, the inhibition induced by $CdCl_2$ was already significant after two hours and remained constant thereafter.

Treatment with AA or $CdCl_2$ did not induce major changes in the morphology of OK cells monolayers at the optical microscopy level (Fig. 4A–D), except that prominent nucleoles were observed following AA exposure in a significant subset of OK cells (Fig. 4D). Confocal microscopy confirmed that the uptake of FITC-albumin was almost abolished in OK cell monolayers exposed to AA (Fig. 4G, H) or to $CdCl_2$ (Fig. 4E, F).

Reversal of the inhibitory effect: AA versus $CdCl_2$

The persistence of the inhibitory effects induced by AA and $CdCl_2$ was assessed using recovery periods of up to six days (Fig. 5). In the case of AA, increased durations of recovery after the initial 24-hour exposure were marked by progressive inhibition of the uptake of FITC-albumin with no recovery at all. These findings were in striking contrast to the progressive normalization of albumin uptake observed during the recovery following $CdCl_2$ exposure.

OK cell viability: Cytotoxic assays, protein synthesis, and glucose reabsorption

Several cytotoxicity assays were performed to rule out the possibility that the inhibitory effects on protein uptake merely resulted from a reduction in the number of OK cells surviving exposure to AA or $CdCl_2$. The integrity of the cell membrane was assessed on the basis of the rapid release of lactate dehydrogenase (LDH) following plasma membrane damage. Compared with cell lysis induced with the detergent Triton X-100, no sizable toxic effects were observed following exposure to AA or $CdCl_2$ (data not shown). The potential cytotoxicity on OK cells also was assessed with the MTT assay, based on the specific reduction of MTT by mitochondrial dehydrogenases in living cells only. This assay also showed no significant difference in terms of cell viability between AA and $CdCl_2$ compared to controls (data not shown). The effect of AA and $CdCl_2$ on protein synthesis was measured by the incorporation of [^{35}S]L-methionine into proteins at 1 or 20 hours, which provided an estimate of synthesis of proteins with short or long half-lives, respectively. As shown in Table 2, no treatment except cycloheximide significantly inhibited protein synthesis, which further supports the lack of major toxic effect related to exposure to AA or $CdCl_2$.

We next tested whether AA or $CdCl_2$ alter glucose reabsorption, by measuring the uptake of AMG, a glu-

Table 1. Michaelis-Menten kinetics of FITC-albumin and Cy5-β_2-microglobulin endocytosis in opossum kidney (OK) cells

	FITC-albumin		Cy5-β_2-microglobulin	
	pmol/L K_m	*pmol/mg protein* V_{max}	*pmol/L* K_m	*pmol/mg protein* V_{max}
Control	477 ± 88	15.2 ± 2.1	844 ± 129	19.8 ± 3.3
$CdCl_2$ 15 μmol/L − 24 h	376 ± 38	11.9 ± 1.5[a]	878 ± 194	9.1 ± 1.5[a]
DMSO 0.1% − 24 h	360 ± 12	14.1 ± 2.8	533 ± 76	21.6 ± 4.5
AA 10 μmol/L − 24 h	394 ± 40	11.7 ± 2.7[a]	394 ± 98	8.2 ± 3.1[a]
AA 20 μmol/L − 24 h	456 ± 30[a]	7.2 ± 2.3[a]	431 ± 51	5.8 ± 1.4[a]
AA 20 μmol/L − 24 h + 24 h	n.d.[b]	1.6 ± 1.1[a]	n.d.[b]	2.9 ± 1.6[a]

The values of the apparent affinity (K_m) and maximum rate (V_{max}) were calculated by the Eadie-Hofstee linearized equation. Abbreviations are: FITC, fluorescin-isothiocynate; DMSO, dimethyl sulfoxide; $CdCl_2$, cadmium chloride; AA, aristolochic acid.

[a] $P < 0.05$ by comparison to paired control values; $N = 3$ in each set of experiments

[b] These values could not be determined accurately because of the low values of the plateau (V_{max})

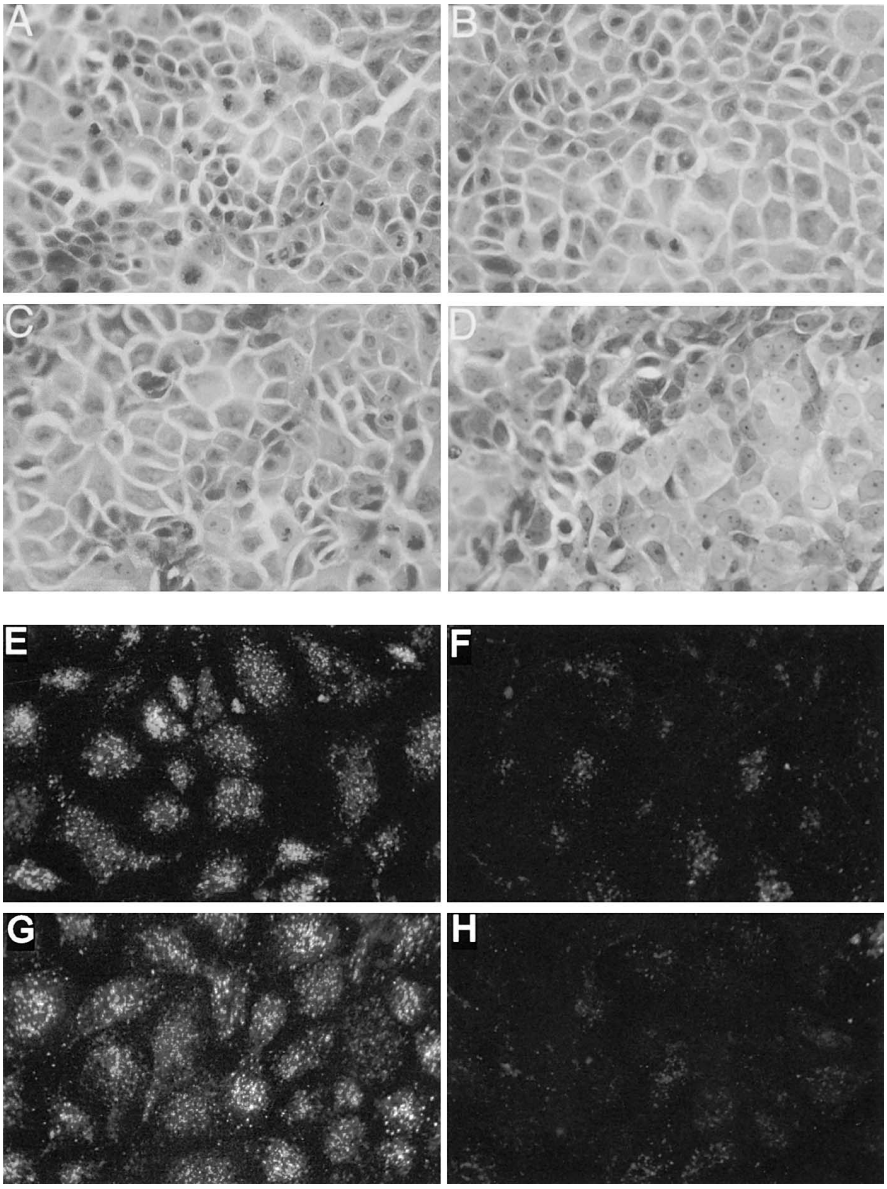


Fig. 4. Effect of a 24-hour exposure to AA or $CdCl_2$ on the morphology of OK cell monolayers and on the level of FITC-albumin endocytosis observed by confocal microscopy. OK cell monolayers were incubated in control conditions (medium alone: *A* and *E*) or exposed for 24 hours to 15 μmol/L $CdCl_2$ (*B* and *F*); 0.1% DMSO (*C* and *G*); 20 μmol/L AA (*D* and *H*). The upper panels (A to D: ×300) show an eosin-hematoxylin staining of fixed OK monolayers. Note the prominent nucleoli in a subset of cells treated with AA (panel D). The lower panels (E to H, ×350) show the confocal imaging of FITC-albumin endocytosis by OK cells following 15-minute exposure to 150 μmol/L FITC-labeled albumin.

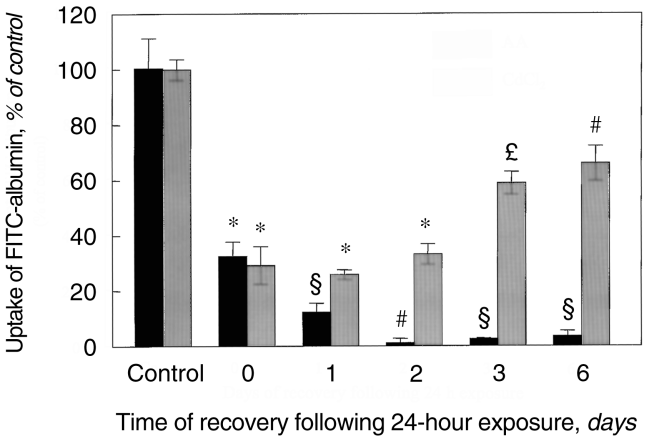


Fig. 5. Recovery following exposure of the OK monolayers to AA (■) and $CdCl_2$ (▩). The cells were preincubated with 0.1% DMSO, 20 μmol/L AA or 15 μmol/L $CdCl_2$ for 24 hours followed by zero, one, two, three, or six days in normal culture medium. The FITC-albumin (0.8 μmol/L) endocytosis assay was performed for 15 minutes immediately after each recovery period. Each column represents the difference between the values observed at 37°C and at 4°C ($N = 3$). *$P < 0.05$ by comparison to control values. §$P < 0.05$ by comparison to control values and values at day 0. #$P < 0.05$ by comparison to control values and values at days 0 and 1. £$P < 0.05$ by comparison to control values and values at day 1.

cose analogue that is a substrate for the Na^+-glucose cotransporter but not for the ubiquitous hexose transporters. A 24-hour exposure of OK cell monolayer to AA or $CdCl_2$ did not modify the uptake of AMG, which also showed Michaelis-Menten kinetics ($K_m = 3138 \pm 234$ mmol/L; $V_{max} = 29.2 \pm 2.3$ mmol/mg protein). This indicates not only that the AMG uptake is not functionally altered by exposure to these compounds, but also that the intracellular to extracellular sodium gradient is maintained, as AMG is cotransported with Na^+ ions.

Detection of DNA adducts in OK cells

The persistence of the AA-induced inhibitory effect on albumin and β_2-microglobulin uptake in the absence of significant modification of protein synthesis or signs of general toxicity is puzzling. We therefore used the ^{32}P-postlabeling method to investigate the possibility that exposure of OK cells to AA permanently alters the DNA by forming agent-specific DNA adducts. The adduct pattern obtained showed the typical adduct profile for AA exposure [37, 38] and consisted of two major spots of (*1*) adenosine adducts of AAI: 7-(deoxyadenosine-N^6-yl)aristolactam I (dA-AAI), and (*2*) guanosine adducts of AAI: 7-(deoxyguanosine-N^2-yl)aristolactam I (dG-AAI), and (*3*) one minor spot of adenosine adducts of AAII: 7-(deoxyadenosine-N^6-yl)aristolactam II (dA-AAII). These purine adducts are identical to those identified previously in several organs of rodents treated with AA as well as in kidney and ureter tissue of patients with CHN [38]. OK cells thus are competent to metabolize AA to the same reactive intermediates leading to DNA adducts identical to those found in vivo. Quantitative analysis shown in Table 3 revealed that adduct level increased linearly with the duration of AA exposure as well as with the AA concentration while they remained unchanged 24 hours after AA removal. Following a recovery period of six days, the adduct level was still significant. This clearly demonstrates a permanent alteration of the DNA by AA. No such bulky adduct spots were observed in the case of exposure to $CdCl_2$ or to the vehicle (DMSO) alone under our assay conditions.

Table 2. Effect of AA and $CdCl_2$ on protein synthesis by opossum kidney (OK) cells

	10^6 CPM/mg protein	
	1 hour	20 hours
Control	22.0 ± 2.4	9.6 ± 1.8
AA 20 μmol/L – 24 h	13.4 ± 2.7	9.4 ± 1.0
$CdCl_2$ 15 μmol/L – 24 h	17.5 ± 1.6	8.8 ± 0.4
Cycloheximide 100 μmol/L – 1h	1.4 ± 0.08[a]	1.0 ± 0.06[a]

Abbreviations are in Table 1.

The cells were treated with AA or $CdCl_2$ followed by 1 hour either in culture medium or 1 hour in the presence of 100 μmol/L cycloheximide. After 2 hours' exposure to [^{35}S]L-methionine, the monolayers were incubated for 1 or 20 hours in culture medium supplemented with an excess of methionine. The count per minute (CPM) values represent the mean ± SEM of triplicate count.

[a]$P < 0.05$ by comparison to control values

Megalin expression in OK cells: Colocalization with albumin and effect of AA

The plasma membrane expression of megalin in OK cells was documented by confocal microscopy (Fig. 6). As already reported [31], megalin appears to function as a receptor for endocytosis of LMWP. Indeed, FITC-albumin added to the incubation medium is rapidly internalized and concentrated within minutes in intracellular vesicles that also express megalin (Fig. 6, yellow emission). Since exposure to AA reduced considerably the rate of internalization of albumin (Fig. 4), we therefore evaluated whether megalin expression was affected by this treatment. Three independent immunoblot experiments demonstrated a significant decrease of megalin expression in OK cells incubated for 24 hours with AA (Fig. 7A). Semiquantitative analysis further showed that exposure to AA induced a 70% reduction in megalin expression (relative to β-actin) when compared with its control (DMSO alone; Fig. 7B).

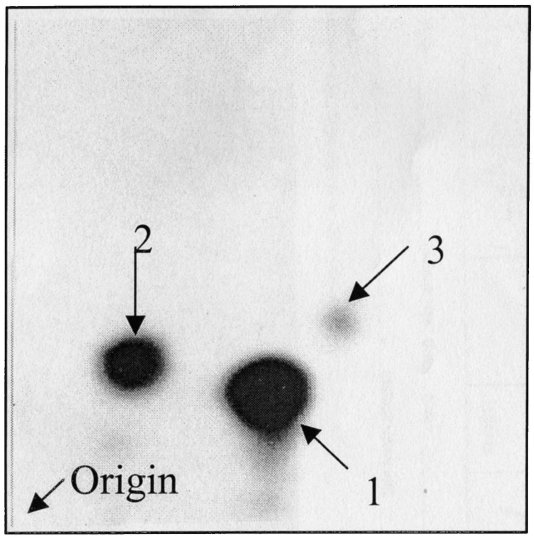
DISCUSSION

The OK cell line was used as a model for PT epithelial cells to substantiate the involvement of AA in the tubular proteinuria, which is a consistent and early feature of CHN. The toxicity of AA was compared with that of $CdCl_2$, a well-characterized PT toxin. A transient exposure of OK cells to either AA or $CdCl_2$ inhibits the

Table 3. Time- and concentration-dependent formation of DNA adducts by AA in OK cells

Exposure to aristolochic acid	RAL/10^7 normal nucleotides		
	dA-AAI (spot 1)	dG-AAI (spot 2)	dA-AAII (spot 3)
20 μmol/L — 15 min	0.11 ± 0.02	0.31 ± 0.01	ND
20 μmol/L — 2 h	0.53 ± 0.03	1.9 ± 0.6	0.16 ± 0.03
20 μmol/L — 14 h	4.2 ± 1.3	24.0 ± 9.2	1.5 ± 0.5
10 μmol/L — 24 h	28.1 ± 1.9	16.0 ± 2.1	3.0 ± 0.4
20 μmol/L — 24 h	55.6 ± 15.0	25.5 ± 0.6	5.6 ± 1.3
20 μmol/L — 24 h + 1 d recovery	58.6 ± 10.6	28.8 ± 1.7	6.2 ± 0.2
20 μmol/L — 24 h + 6 d recovery	7.7 ± 1.3	6.9 ± 1.9	0.74 ± 0.19

The relative adduct labeling (RAL) values of the specific AA-adduct spots: spot 1, dA-AAI [7-(deoxyadenosine-N^6-yl)aristolactam I]; spot 2, dG-AAI [7-(deoxyguanosine-N^2-yl)aristolactam I]; spot 3, dA-AAII [7-(deoxyadenosine-N^6-yl)aristolactam II] represent the mean ± SEM of triplicate ^{32}P-postlabeling analysis. ND is not detectable.



endocytic mechanism of protein reabsorption. However, while exposure to $CdCl_2$ results in a transient effect, exposure to AA results in a permanent alteration of albumin endocytosis with no sign of recovery for up to six days. The functional alterations induced by AA in OK cells are mirrored by a dose-dependent formation of AA-specific DNA adducts and a significant decrease in the expression of megalin, the receptor involved in albumin endocytosis.

The present study confirms that albumin and β_2-microglobulin are reabsorbed in OK cells by receptor-mediated endocytosis (Fig. 1A and Table 1) [30, 39], the two proteins apparently competing for the same receptor (Fig. 1B, C). The latter has been identified as a multimeric complex of cubilin and megalin, located both in the brush border of PT epithelial cells [23, 40, 41] and also in OK cells [31]. Our immunoblotting and immunostaining studies confirmed the expression of megalin in OK cells (Figs. 6 and 7). Furthermore, confocal microscopy performed after addition of FITC-albumin to the incubation medium showed its colocalization with megalin, first at the cell margins and within minutes, in intracellular vesicles (Fig. 6).

A 24-hour exposure of OK cell monolayers to AA or to $CdCl_2$ similarly inhibited the endocytic mechanism of protein reabsorption. These inhibitions are characterized by a reduced V_{max} with no change in K_m (Table 1), indicating that AA or $CdCl_2$ decreased the number of recycling receptors able to internalize LMWP, which could actually correspond to a reduction of plasma membrane receptors, of their internalization or of their recycling, with no change in their affinity. These effects do not result from nonspecific toxicity, since both cell viability and protein synthesis remained unaltered. Also, the lack of inhibition of Na^+-glucose cotransport implies that the low intracellular Na^+ concentration is maintained, hence the Na^+,K^+-ATPase must remain active in OK cells that have been exposed to AA or $CdCl_2$. However, a striking difference between AA and $CdCl_2$ was observed when examining recovery after removal (Fig. 5). When AA was removed and the incubation period prolonged up to six days, the uptake of FITC-albumin remained inhibited, suggesting permanent alteration in the endocytic machinery. In contrast, removal of $CdCl_2$ led to a progressive, although not complete, restoration of protein uptake over six days. Thus, unlike AA, $CdCl_2$ does not induce a permanent alteration of protein reabsorption in OK cells.

The toxic effects of $CdCl_2$ have been mainly related to increased catabolism leading to decreased expression and activity of several membrane proteins [42, 43] including the vacuolar H^+-ATPase required for receptor mediated endocytosis. Our observations of endocytosis recovery following $CdCl_2$ exposure (Fig. 5) indeed are compatible with increased catabolism [42, 43]. In contrast, the lack of recovery following AA exposure suggests that its toxicity is related to the sustained decrease in one (or a few) protein(s) involved in receptor-mediated endocytosis. Such an effect might be overlooked when total cell protein synthesis is measured since it would only represent a low percentage of this total. However, assessment of megalin expression by repeated immunoblot analyses of OK cells exposed to AA showed a significant (~70%) decrease as compared with β-actin (Fig. 7). Of note, this effect of AA on megalin expression in OK cells was not observed for other proteins involved in receptor-mediated endocytosis such as the vacuolar H^+-ATPase or the endosomal CLC-5 channel [35], whose expression levels were unchanged (data not shown).

By analogy to what has been described in the kidneys of patients with CHN [38], the persistence, and even

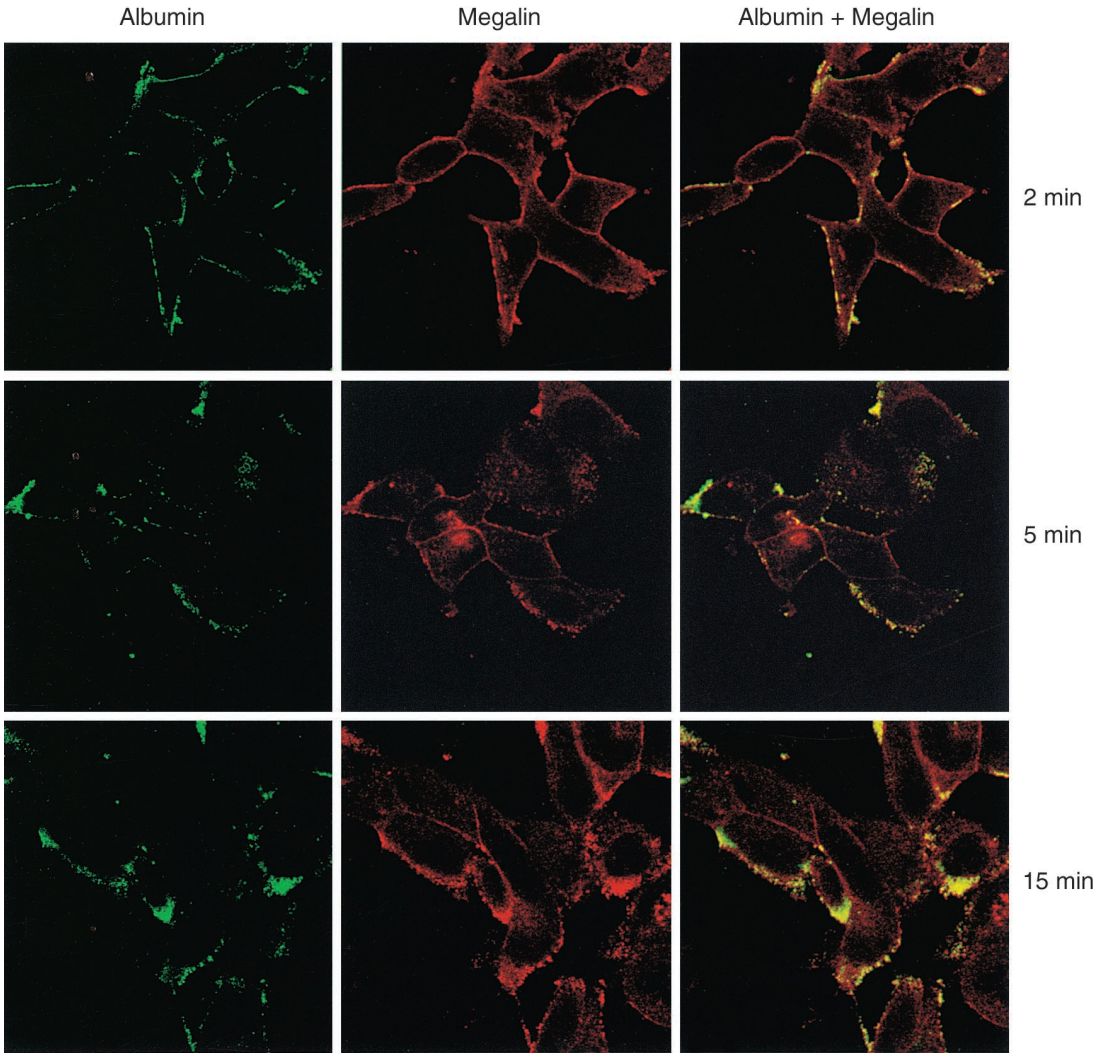


Fig. 6. Albumin endocytosis and endogenous expression of megalin in OK cells: confocal microscopy. Albumin endocytosis was studied after incubation of OK cell monolayers with 150 μmol/L albumin-FITC and imaging by confocal microscopy after 2, 5, and 15 minutes. Fluorescent endocytosis vesicles appear near the margins of the cells after the 2-minute incubation and extend deeper into the cytoplasm after 5- and 15-minute incubation. Endogenous megalin in OK cells was detected by immunofluorescence with a TRITC-labeled secondary antibody. A characteristic chicken-wire pattern, delineating plasma membrane reactivity of megalin, is observed at two minutes. A progressive internalization of the labeling is seen after 5 and 15 minutes after incubation with albumin. Examination of the colocalization between megalin (red) and albumin (green) at the three time points reveals that a substantial fraction of albumin is colocalized with megalin (yellow emission), first at the plasma membrane and later in intracellular vesicles. The confocal settings were identical for the three time points shown. All panels ×200.

aggravation, of the toxicity of AA for several days (Fig. 5) might be due to DNA alteration. We therefore investigated whether AA induced a persistent alteration of DNA in OK cells. Such a process indeed might impair the synthesis of a key component of receptor-mediated endocytosis such as megalin. Thus, three characteristic spots of DNA adducts for AA were observed in OK cells exposed to AA but not in controls, nor after $CdCl_2$ exposure. The level of AA-DNA adducts increased with the concentration of AA, remained stable 24 hours after AA removal, and was still high six days after removal (Table 3). These observations are consistent with the persistence of AA-DNA adducts in various organs of rats exposed to AA [38] and tissues of patients with CHN [14]. They also might be relevant to understand the progression of the disease despite interruption of the AA-containing regimen in CHN patients [14].

The inhibition of protein uptake by OK cell monolayers required a sustained incubation with AA for at least 14 hours, that is, a time when AA-DNA adducts are already detected. Furthermore, both alterations exhibit a similar concentration dependence and persist after removal of AA. It is therefore tempting to suggest a causal relationship between exposure to AA, specific DNA damage, and cell-specific alterations at the transcription level, particularly in the decreased synthesis of megalin.

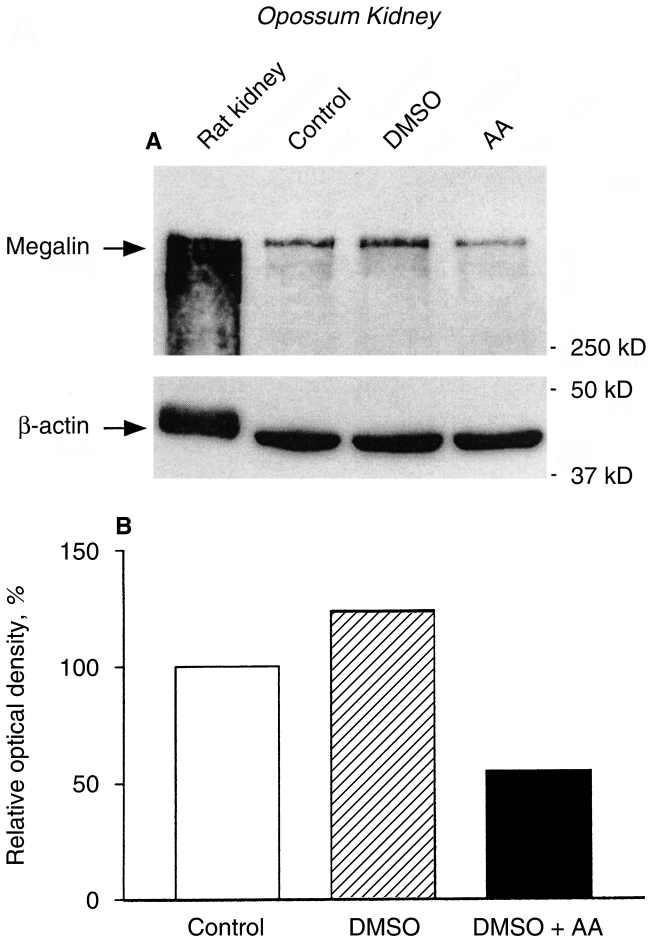


Fig. 7. Expression of megalin and exposure to aristolochic acid in OK cells: immunoblot analysis. (*A*) Membrane extract from rat kidney cortex (positive control) [35] and lysates from OK cells (30 μg protein/lane) incubated in control conditions or for 24 hours with 0.1% DMSO alone or containing 20 μmol/L AA were loaded on a 5% polyacrylamide gel. The blot was probed with sheep anti-megalin antibodies and, after stripping, with a monoclonal antibody against β-actin. A >500 kD band, corresponding to the predicted molecular mass of megalin, is identified in rat kidney cortex and OK cell lysates. Examination of the relative signal intensities shows that the expression of megalin is decreased in OK cells that have been exposed to AA for 24 hours. The film is a representative immunoblot of three independent experiments. (*B*) Densitometry analysis. Compared with control OK cells, the signal intensity for megalin is slightly increased (+24%) when the cells are incubated with DMSO alone, whereas it is decreased by half (−45%) when cells are incubated with AA in DMSO. The relative optical densities were obtained in duplicate and normalized over β-actin.

Since our goal was to specifically address an early sign of AA toxicity in a cell culture system that does not allow prolonged exposure to a toxic agent, we needed to expose OK cells to rather high doses of AA for 24 hours. Therefore, the dose of AA used in this study was approximately one order of magnitude higher than that absorbed daily by CHN patients [4]. As mentioned before, this exposure was mirrored by detection of specific AA-DNA adducts. The rather selective toxicity of AA for the kidney and PT cells deserves further attention. Recent evidence from our group indicates that AA is concentrated within PT cells via the basolateral organic anion transporter (OAT1) [44], which exchanges organic anions like paraaminohippurate (PAH) (and AA) for α-ketoglutarate. This mechanism of tertiary active transport (relying on a Na^+-α-ketoglutarate cotransport located in parallel) concentrates AA within PT cells and the high intracellular concentration so achieved might account for its selective toxicity. Thus, despite differences in exposure, intracellular concentrations of AA may be similar in OK cells and PT cells of CHN patients, as suggested by similar early manifestations of toxicity.

Interspecies differences in the metabolism of AA have been reported in mammals [45]. In human, the urinary metabolites are aristolactam I and II (corresponding to AAI and AAII, respectively), resulting from the formation of a monoamide cycle (lactam). Interestingly, similar AA-DNA adducts (that is, aristolactam I and II derivatives) were observed in human kidney and OK cells, which suggests a similar metabolism within PT and OK cells. Finally, although our data show that AA alone can induce PT cell dysfunction, it is possible that other compounds absorbed by CHN patients are involved in the rapid progression of the disease. Since PT cells concentrate and excrete xenobiotics by several mechanisms [46], it is possible that other drugs simultaneously taken by CHN patients have influenced the rate of luminal AA exit, hence its intracellular accumulation.

In conclusion, our observations substantiate the involvement of AA in the early PT dysfunction observed in CHN and further suggest that decreased megalin expression accounts, at least in part, for the inhibition of luminal protein reabsorption by PT cells. In addition to their potential role in mutagenesis and carcinogenesis [47, 48], AA-DNA adducts might impair a physiological process such as receptor-mediated endocytosis.

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